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A Presenilin-like protease associated with *Plasmodium falciparum* micronemes is involved in erythrocyte invasion

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Abstract

We describe identification of a *Plasmodium falciparum* microneme protease involved in RBC invasion. From the yeast two-hybrid screening of a *P. falciparum* cDNA library, we have identified a 47 kDa membrane protein that interacted with the 5ABC domain of human RBC band 3. This protein shared homology with a presenilin-type aspartyl protease, the signal peptide peptidase (SPP). An antibody raised against a predicted exposed region of this protein reacted specifically to a single band of ~47 kDa in the *P. falciparum* protein extract. Immunofluorescence microscopy suggested that this protein co-localized with the microneme protein EBA-175 in schizonts, and immunoelectron microscopy established that it is primarily localized to micronemes in merozoites. Functional characterization of PfSPP, *Plasmodium falciparum* signal peptide peptidase, demonstrates that an antibody to PfSPP blocks RBC invasion by *P. falciparum* *in vitro*. Native and recombinant PfSPP bound directly to the 5ABC domain of band 3 in solution and the binding of PfSPP to RBCs was chymotrypsin-sensitive, but trypsin and neuraminidase-resistant. Together, these results suggest that host band 3 interacts with PfSPP during RBC invasion presumably following parasite microneme discharge. PfSPP is the first microneme-associated intramembrane aspartyl protease identified in the apicomplexan parasites that interacts with a major transmembrane receptor on host erythrocytes.

Keywords

Plasmodium falciparum; microneme protein; intramembrane aspartyl protease; erythrocyte invasion; band 3

1. Introduction

The clinical manifestations of malaria are directly linked to the blood-stage lifecycle of *Plasmodium* parasites, in which the parasites propagate asexually within host red blood cells (RBCs). Each asexual multiplication cycle follows the invasion of RBCs by merozoites, which are released into the blood stream from the rupture of host cells. It is believed that specific interactions between erythrocyte and parasite proteins play a central role during merozoite attachment on the RBC surface, and subsequent penetration of the parasite into the RBC in the blood stream [1,2]. Host-parasite protein interactions are also thought to be crucial to sustain signal transduction pathways that modulate timed molecular events required for RBC invasion. Thus, there has been a great interest to uncover receptor-ligand interactions that occur particularly between the human host cell and *Plasmodium falciparum*, the lethal malaria species for human infections. In *P. falciparum*, the RBC invasion mechanism is more complex

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than in other *Plasmodium* species as two unique RBC invasion phenotypes have been identified in the field isolates [3–5] and laboratory strains [6–9]. These *P. falciparum* phenotypes have been broadly defined by the parasite's preference to sialic acid residues on the host RBC surface at an unidentified time point in the course of the invasion process. To date, only a handful of receptor-ligand interactions between human RBCs and *P. falciparum* have been definitively identified. RBC sialoglycoproteins, glycophorin A and glycophorin C, have been shown to interact, respectively, with *P. falciparum* EBA-175 (175 kDa erythrocyte binding antigen) [10–12] and EBA-140 (also known as BAEBL) [13–16]. Previously, we have shown that host band 3 interacts with *P. falciparum* MSP1 (merozoite surface proteins 1), a GPI-anchored merozoite surface protein, and MSP9, a peripheral merozoite surface protein, as a co-ligand complex [17–19]. The RBC antigen Kx has been suggested to interact with *P. falciparum* AMA-1 (apical membrane antigen-1) [20]. In addition, several hypothetical human RBC receptors designated as Y [21], Z [22], E [23], and X [24,25] have been proposed to interact, respectively, with *P. falciparum* RH1 (reticulocyte-binding protein homologue 1), RH2b, EBA-181 (also known as JESEBL), and an unidentified merozoite protein. Several other *P. falciparum* proteins either embedded in the merozoite membrane or secreted into the merozoite surface from the apical organelles have been suggested to participate in RBC invasion, but neither their specific interactions nor function have been established [1].

Here, we describe the identification and initial characterization of a 47 kDa *P. falciparum* integral membrane protein from the *P. falciparum* cDNA library screen. The 47 kDa protein is homologous to a presenilin-type intramembrane aspartyl protease, the signal peptide peptidase (SPP). Golde and coworkers [26] recently reported that a recombinant form of this protease, termed malaria signal peptide peptidase (mSPP), expressed in mammalian cells exhibits intramembrane proteolytic activity resembling that of a known human signal peptide peptidase. Our protein co-localization and electron microscopic studies demonstrate that this protein is localized to micronemes and apical surface in mature parasites. The microneme protease interacted with the 5ABC domain of RBC band 3 in solution, and a mono-specific antibody against this protein blocked the RBC invasion by *P. falciparum* in culture. We have designated the *Plasmodium falciparum* signal peptide peptidase as PfSPP. To our knowledge, PfSPP is the first identified apicomplexan intramembrane aspartyl protease that is localized to an apical organelle and interacts with the host erythrocytes.

2. Materials and methods

2.1. Parasite culture and solubilized parasite proteins

The *P. falciparum* strain 3D7 was maintained in continuous culture in a 5% suspension of fresh type O+ human erythrocytes in RPMI 1640 at 37°C under 5% CO₂, 5% O₂, and 90% N₂ by the method of Trager and Jensen [27]. Ring-stage parasites were synchronized by using 5% Sorbitol treatment and late-stage parasites were enriched to >95% by centrifugation using 63% (v/v) Percoll as described [17]. The parasite protein extract was prepared by solubilizing an enriched fraction of mature parasites with an extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 0.5% BSA) supplemented with 2 µg/ml Aprotinin, 1 µg/ml of Leupeptin, Pepstatin A, Bestatin, 10 mM PMSF, and a cocktail of protease inhibitors (Roche). The mixture was kept on ice for 1 h and centrifuged at 12,000 g for 20 min at 4°C. The supernatant was stored at –80°C in aliquots and used in the antibody characterization and binding assays.

2.2. Recombinant Proteins

The PfSPP gene fragment encoding amino acids 220–260 (designated as the R3 region) was amplified by PCR from *P. falciparum* (3D7) genomic DNA and cloned into pMAL-c2x (NE Biolabs). The PCR primers used were 5'-CGCGAATTCGTATATGATATTTTCTGG-3'

(sense, *Eco*RI) and 5'-CGCTCTAGAACCAAGCATACTGTAATG-3' (antisense, *Xba*I). The recombinant PfSPP/ER domain was expressed in *Escherichia coli* DH5α as a fusion to the maltose binding protein (MBP). MBP-PfSPP/ER was affinity-purified on amylose resin. The 5ABC peptide corresponds to residues 720–761 (GMPWLSATTVRSVTHANALTVMGKASTPGAAAQIQEVKEQR I) of human band 3. Affinity purified GST-5ABC and Trx-5ABC fusions were prepared as described [18]. Trx-5ABC eluted from Ni-NTA beads was dialyzed against PBS (pH7.4) and purity of all recombinant proteins was determined by Coomassie stained gels.

2.3. Mono-specific anti-PfSPP antibodies

Anti-peptide antibodies against the PfSPP/ER region were produced by the UIC Research Resources Center. A short peptide corresponding to residues 240–258 (EAPVKLLFPVSSDPV HYSM) of *P. falciparum* (3D7) PfSPP was synthesized with an additional cysteine residue at the N-terminus and conjugated to Keyhole Limpet Hemocyanin, KLH, through a disulfide bond. Peptide-specific antibodies raised in rabbits were harvested by affinity purification of serum on the cyanogen bromide-immobilized peptide. The affinity-purified antibodies, stored at 4°C in PBS, were tested for specific reactivity against recombinant PfSPP/ER and native *P. falciparum* PfSPP by Western blotting.

2.4. Immunofluorescence microscopy

Synchronized *P. falciparum* (3D7) schizonts were smeared and air dried on glass slides and fixed with 100% methanol for 30 min at –20°C. Slides were incubated for 2 h with a mixture of two antibodies containing affinity purified mono-specific anti-PfSPP rabbit Abs (1:1000) and anti-EBA-175 rat pAb (1:1000; MR4), or anti-MSP1 mouse mAb 5.2 (1:1000; MR4), or anti-RAP1 mouse mAb (1:1000; MR4). After three washings in PBS-T (0.1% Tween-20), samples were incubated for 1 h with appropriate secondary Abs (Molecular Probes) conjugated with either Alexa Fluor 488 (green fluorescence) or Alexa Fluor 594 (red fluorescence). Dual-color fluorescence images were captured using a microscope (Zeiss LSM510, Germany) equipped with a digital camera at 100X magnification.

2.5. Immunoelectron microscopy

Late-stage schizonts (40–48 h post invasion) enriched from a synchronized *P. falciparum* culture were washed in RPMI 1640, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 1 h at 4°C in 0.1 M sodium phosphate buffer (pH 7.2), and embedded in White London Resin. Ultra-thin sections were blocked in PBS containing 1% BSA and incubated with affinity purified anti-PfSPP pAb diluted in the above-mentioned solution. Samples were washed and incubated with secondary antibodies conjugated with gold particles (10 or 15 nm diameter) at a 1:10 dilution for 1 h. Labeled sections were stained with uranyl acetate and lead citrate, and observed using a Philips FEI Tecnai F30 transmission electron microscope at 300 kV at the University of Chicago EM facility.

2.6. RBC invasion inhibition assay

A fraction enriched with late-stage *P. falciparum* trophozoites was washed twice with RPMI 1640 and returned to the culture by incubating the parasites with fresh RBCs in a 96-well microplate (final, 3% hematocrit, 2% parasitemia). An aliquot of affinity purified anti-PfSPP Abs and pre-immune IgG were added to the culture medium as the inhibitor to give 0, 12.5, 25, 50, and 100 µg/ml final concentration in a volume of 200 µl. The sample with no antibodies, PBS only, served as a negative control. Rabbit pre-immune serum was used at 10% dilution as another negative control. Parasite samples were incubated for 24 h under standard culture conditions and assays were carried out in triplicates. Each sample was analyzed by counting the ring-stage parasites in Giemsa-stained thin smears. Approximately 2,000 RBCs were

counted for each sample to quantify parasitemia, from which the percent inhibition of invasion was calculated relative to the control sample with no Abs: (parasitemia for no Abs control – parasitemia for sample containing Abs) / parasitemia for no Abs control $\times$ 100%. Data are presented as mean $\pm$ s.d.

2.7. RBC binding in suspension

Trophozoites enriched from a synchronized *P. falciparum* culture were returned to the culture containing only the complete culture medium and allowed to mature for 16 h to form schizonts and merozoites. The parasites were left in the culture flask to lyse and release proteins into the culture supernatant. The resulting culture was centrifuged at 12,000 g for 20 min or 40,000 g for 15 min at 4°C. The culture supernatant and pellet were analyzed by Western blot using anti-PfSPP pAbs. The culture supernatant prepared at 12,000 g was stored at –80°C in aliquots and used in subsequent RBC binding assays. A 500 μ l sample of *P. falciparum* culture supernatant was added to normal, trypsin-treated, chymotrypsin-treated, or neuraminidase-treated intact human RBCs (50 μ l packed volume) for 1 h at room temperature. The cells were centrifuged at 12,000 g for 30 s through a layer of silicone oil (500 μ l). Proteins bound to RBCs were eluted by incubating the RBCs in 1.5 M NaCl (20 μ l) for 30 min at room temperature. Salt-eluted proteins were analyzed by SDS-PAGE followed by the Western blotting using anti-PfSPP Abs. The pretreatment of intact RBCs with enzymes was carried out as described [17]. To examine the inhibitory effect of 5ABC on protein binding, soluble GST-5ABC (40 μ M) was added to the normal RBC sample. The GST (40 μ M) protein served as a negative control.

2.8. Solution binding assays

Recombinant protein binding: The MBP-PfSPP fusion conjugated to amylose beads was incubated at 4°C overnight (350 μ l final volume) with soluble Trx-5ABC in the binding buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.5 mg/ml BSA). Beads were sedimented by low speed centrifugation and washed three times with PBS containing 0.1% Tween 20 (PBS-T). Trx-5ABC bound to the beads was detected by Western blotting using anti-His-HRP mAb (Santa Cruz Biotechnology). MBP on Ni-NTA beads and soluble Trx were used as negative controls.

Native protein binding: GST-5ABC and GST conjugated to GSH beads (30–60 μ l) were incubated with the solubilized *P. falciparum* proteins (200 μ l) in the binding buffer (PBS, 1 mM EDTA, 0.1% w/v Triton X-100, and 0.5 mg/ml BSA) at 4°C overnight (600 μ l final volume). The resulting beads were sedimented by centrifugation, washed three times with binding buffer without BSA, and analyzed by SDS-PAGE followed by the Western blotting using affinity-purified anti-PfSPP pAbs.

3. Results

3.1. Identification of a Presenilin-like peptidase in *P. falciparum*

Earlier, we reported a screen of a *P. falciparum* (3D7) cDNA library in a yeast two-hybrid system using a peptide (5ABC) patterned on human RBC band 3 as bait [18]. This screen identified a novel interaction between band 3 and the *P. falciparum* merozoite surface protein 9 (MSP9), which existed as a co-ligand complex of MSP9-MSP1 [19]. We detected another *P. falciparum* gene product that interacted strongly with the 5ABC peptide in the yeast two-hybrid assay under the highest stringency conditions. The sequence of the cDNA insert in the yeast vector was linked to a *P. falciparum* gene (1,218 bps) designated as PF14_0543 in the PlasmoDB encoding a hypothetical protein of 46.9 kDa (NP_702432). Transcriptome analyses of several *P. falciparum* strains compiled in the PlasmoDB indicated that PfSPP is transcribed at a relatively high level at the late stage (Trophozoite/Schizont) of parasite intraerythrocytic

life cycle. The proteome data compiled in the PlasmoDB suggest that PfSPP is expressed in merozoites.

The *P. falciparum* PfSPP orthologues exist in different *Plasmodia* species including *P. vivax*, *P. knowlesi*, *P. yoelii*, *P. berghei*, and *P. chabaudi*, other apicomplexa species such as *Cryptosporidium parvum* and *Toxoplasma gondii*, and many other single and multicellular eukaryotes (OrthoMCL DB, <http://orthomcl.cbil.upenn.edu>). However, no PfSPP paralogue was found in *P. falciparum* indicating the existence of a single gene encoding PfSPP in the parasite genome. Blast search analysis showed that PfSPP shares homology with the Presenilin-type intramembrane aspartyl proteases functionally identified in higher eukaryotes, including humans. In fact, a phylogenetic analysis of the orthologues showed that *P. falciparum* PfSPP is homologous to human and mouse Signal Peptide Peptidase (SPP) and human Presenilin (Fig. 1A).

Primary structure analysis of PfSPP revealed conservation of aspartate-containing catalytic site motifs YD (amino acids 221–222) and LGLGD (259–263) that are required for the activity of Presenilin-type intramembrane aspartyl proteases [28] (Fig. 1B). The C-terminal PAL (335–337) motif [29] essential for a proper conformation of the active site is also present in PfSPP. An uncleaved signal-anchor rather than a signal peptide sequence is predicted for PfSPP by SignalP 3.0 software. Analysis of the secondary structure of PfSPP, predicted using various topology-modeling algorithms, suggested that the protein has 8–10 transmembrane (TM) helices (Fig. 1B). TMPred and TMHMM-2.0 programs predicted eight TM domains with preference for a cytosolic orientation of the N-terminus. Phobius predicted ten TM domains with a cytosolic N-terminus. SOSUI predicted nine TM helices without a particular preference for an orientation in the membrane. However, only the later three topology models showed intramembrane active site motifs YD and LGLGD in the two adjacent TM domains as required for the Presenilin-type proteases. Thus, the TMPred model was not considered further in this study. In all three topology models, the PAL motif was at the boundary of the adjacent TM region in proximity to the two TM helices forming the catalytic site. In fact, the PAL motif found in PfSPP is followed by Leu-338 to create the PALL (335–338) motif typically conserved in SPP and is similar to the PALP motif conserved in Presenilin [28]. The SOSUI-predicted topology model shows nine TM helices with the N- and C-termini to be in an opposite orientation. For PfSPP to function as a SPP that can catalyze the cleavage of type II membrane proteins, the two adjacent TM helices forming the active site must be in an orientation that permits the polypeptide connecting the two TM helices to adopt an extracytosolic conformation as shown in Fig. 1B (d). An intramembrane catalytic site oriented in the opposite direction due to the inverted orientation of TM helices in the membrane would produce the Presenilin cleaving activity only for type I membrane proteins. Therefore, the three topology models predicted by TMHMM-2.0, Phobius, and SOSUI combined with the identified motifs suggest that the *P. falciparum* PfSPP has characteristics of Presenilin-like SPP rather than Presenilin. Recently, a recombinant form of *P. falciparum* PfSPP expressed in mammalian cells showed some SPP activity using a SPP-cleavable type II membrane domain as the substrate in a cell-based assay, and this activity was inhibited by known SPP inhibitors [26].

The cDNA insert linked to PfSPP in the yeast two-hybrid screen encoded the C-terminus of the protein (amino acids 177–406) containing two or three putatively exposed regions (Fig. 1B). One extracytosolic region, ER, contained 18–26 amino acid residues depending on the topology model, whereas the other two regions contained only a few residues. To establish the existence of the hypothetical PfSPP, anti-peptide antibodies were raised in rabbits against a segment of putative ER of PfSPP. The ER domain of PfSPP was produced as a fusion to the maltose binding protein (MBP-PfSPP/ER) by PCR cloning (Fig. 2A). Mono-specific polyclonal antibodies (pAbs) that were affinity-purified using an immunogen-peptide column reacted specifically to recombinant PfSPP/ER domain in the Western blot assay (Fig. 2B, lane

2). These antibodies reacted to a specific protein migrating at ~47 kDa as a single band in the Western blot of *P. falciparum* (3D7) protein extract containing a mixture of native parasite proteins (Fig. 2B, lane 4). These results suggest that *P. falciparum* PfSPP is expressed during the blood stage of parasite development.

3.2. Localization of PfSPP

To investigate the expression characteristics of PfSPP in mature parasites, we carried out protein co-localization studies using the anti-PfSPP pAbs. The indirect immunofluorescence assay (IFA) using *P. falciparum* schizonts showed that PfSPP co-localized with EBA-175 (175 kDa erythrocyte binding antigen) (Fig. 3). There appears to be some overlap of immunofluorescence with RAP1 (rhoptry-associated protein 1) presumably due to the close proximity of the two organelles, but there was no overlap with MSP1 (merozoite surface protein 1) (Fig. 3). These results suggest that PfSPP is expressed in the micronemes of mature parasites. Since PfSPP was detected in the merozoites in a recent proteomics study [30], we performed labeling of PfSPP using the immunogold electron microscopy. While the control preimmune sera did not show any labeling of the merozoites (Fig. 4A), the anti-PfSPP antibody showed specific labeling of the micronemes and the apical surface of merozoites (Fig. 4B). Consistent with the IFA results, the immunogold particles were not detected in the parasite rhoptries. These results suggest that PfSPP appears to be expressed in micronemes and on the apical surface.

3.3. Blocking RBC invasion by anti-PfSPP antibodies

We investigated a potential function of PfSPP in RBC invasion. First, we evaluated whether the mono-specific anti-PfSPP Abs could block the *P. falciparum* invasion of RBCs in culture. The invasion inhibition assay, as previously described [18], showed that the PfSPP Abs added to the culture medium block *P. falciparum* from invading RBCs (Fig. 5A). The invasion blocking effect was concentration-dependent showing over 45% inhibition of invasion at 100 µg/ml antibody concentration as compared to the PBS (no antibodies) control sample. Anti-PfSPP antibodies did not affect the maturation of parasites since the trophozoites and schizonts did not accumulate in the assay samples (data not shown). Preimmune sera added at 10% dilution showed $2.0 \pm 1.1\%$ inhibition of invasion, which is considered relatively insignificant compared to the anti-PfSPP Abs samples. Total IgG isolated from preimmune sera and added up to 100 µg/ml also showed insignificant inhibitory effect on parasite invasion ($2.6 \pm 0.4\%$). Therefore, it is unlikely that the inhibition of RBC invasion by anti-PfSPP Abs is simply a consequence of steric interference. Together, these results suggest that PfSPP is present in the intact merozoites with exposed polypeptides permitting interactions with the anti-PfSPP Abs in the culture medium and could play an essential role in RBC invasion.

3.4. Binding of native PfSPP to RBCs

To carry out a RBC-binding assay in solution using native PfSPP, we prepared a spent culture supernatant of *P. falciparum* containing native PfSPP. As evident in the Western blot (Fig. 5B), the full-length PfSPP was found in the supernatant fraction obtained by ultracentrifugation at 40,000 g (lane 1) and high-speed centrifugation at 12,000 g (lane 2). Presumably, the PfSPP present in the supernatant fractions was mostly present in small vesicular form as we observed a significant reduction of PfSPP upon ultracentrifugation (lane 1). Consistent with this observation, a relatively higher amount of full-length PfSPP was associated with the pellet fractions in both centrifuged samples (Fig. 5B; lanes 3 and 4). These biochemical properties are consistent with the primary structure of PfSPP consisting of multiple predicted membrane-embedded domains (Fig. 1B).

To further investigate a potential interaction between PfSPP and human RBCs, we carried out a solution-binding assay using the *P. falciparum* culture supernatant prepared by high-speed

centrifugation (12,000 g). Western blotting of the RBC sample using anti-PfSPP Abs showed that native PfSPP associated with intact RBCs (Fig. 5C, lane 1). When soluble GST-5ABC was added to the binding assay, the binding of PfSPP to RBCs was reduced with 94% reduction, which was qualified by densitometry (lane 3). To determine the enzyme-sensitivity of the receptor responsible for PfSPP binding to intact RBCs, we used human RBCs pretreated with trypsin, chymotrypsin, and neuraminidase in the binding assays as previously described [17]. Western blotting showed that the association of native PfSPP to RBCs was reduced in the chymotrypsin-treated RBCs with 84% reduction (lane 5), while it remained relatively unchanged in the trypsin-treated (lane 4) and neuraminidase-treated (lane 6) RBCs as compared to the normal RBC sample (lane 1). It is noteworthy that the extracellular regions of human band 3 in intact RBCs are sensitive to chymotrypsin, but remain resistant to the other two enzymes [17]. Together, our results suggest that the host band 3 receptor is involved in the association of native PfSPP to human RBCs in suspension.

3.5. Direct binding of PfSPP to the 5ABC domain of band 3

Since native PfSPP is insoluble, our RBC binding assay could not distinguish whether PfSPP bound directly to intact RBCs or through other proteins that are likely to be present in the vesicles containing PfSPP. Moreover, a direct involvement of host band 3 in mediating direct association of PfSPP to RBCs was also not conclusive. To address this issue, we carried out a bead-binding assays using native PfSPP solubilized in the nonionic detergent Triton X-100 (*P. falciparum* protein extract) and GST-5ABC beads. Western blot analysis showed that the detergent-solubilized *P. falciparum* PfSPP bound specifically to the 5ABC domain of band 3 immobilized on beads (Fig. 5D). In addition, we tested direct interaction of PfSPP with a soluble fragment of band 3 using purified recombinant proteins, MBP-PfSPP/ER (conjugated to beads) and Trx-5ABC (soluble protein). The 5ABC domain of band 3 interacted specifically with the PfSPP/ER domain in the Western blot assay (Fig. 5E). These results demonstrate that the putative ER3 segment of parasite PfSPP binds directly to the 5ABC region of RBC band 3 (Fig. 6). Together, these data support our evidence that native PfSPP interacts with human RBCs presumably during *P. falciparum* invasion.

4. Discussion

In this study, we show that PfSPP, a Presenilin-like *P. falciparum* intramembrane protease, is localized in the micronemes and the apical surface. Our immunofluorescence microscopy evidence suggests that PfSPP is expressed in the micronemes of mature parasites, and immunoelectron microscopy results indicate that this microneme protein appears to be expressed in micronemes and on the apical surface. Furthermore, PfSPP interacts with the intact RBC membrane through a trypsin and neuraminidase-resistant but chymotrypsin-sensitive RBC protein, most likely band 3.

Earlier studies by Dvorak [31], Bannister [32,33], Aikawa [34], Miller [35], and their co-workers have revealed that RBC invasion by the *Plasmodium* malaria species occurs in the order of seconds through a stepwise process: (a) an initial weak attachment of merozoites to the RBC surface, (b) reorientation of the merozoite, (c) formation of a tight junction between the parasite anterior end and the RBC membrane with the secretion of apical organelles, (d) movement of the merozoite into the RBC by invaginating the RBC plasma membrane that eventually becomes the parasitophorous vacuole membrane, (e) movement of the tight junction towards the parasite's posterior pole, (f) closure of the invasion pit on the RBC membrane, and (g) internalization of the parasite within the parasitophorous vacuole in the RBC cytoplasm. While such a landmark description of the RBC invasion process has provided a conceptual framework, many recent studies have unveiled significant features of this invasion process at the molecular level. These studies identified receptors and/or ligands that mediate crucial host-

receptor interactions [1,2,36], proteolytic processing and proteases [37,38], signaling pathways [39], and a parasite motor protein associated with RBC invasion [40–42]. Nonetheless, there is still much that remains to be understood about the seemingly complex RBC invasion process. For example, elucidation of the mechanism by which apical organelles are discharged from merozoites and specific details of the moving junction that is formed between the invading parasite and RBC will be important future inquiries. Similarly, deciphering the mechanism used by malaria parasites to selectively recruit RBC and parasite proteins to form the invaginating PVM could provide important insights into the development of new malaria drugs and vaccine.

During RBC invasion by the malaria parasite, it is believed that two apical organelles, micronemes and rhoptries, rapidly secrete their contents when the merozoite apical end comes in close proximity to the host cell membrane. Many parasite proteins that are constituents of the secreted material are thought to play essential roles in the RBC invasion process. In *Toxoplasma gondii*, an apicomplexan related to *Plasmodia*, the secretion of organelles is sequential where micronemes discharge first in association with the tight junction formation. This is followed by the discharge of rhoptries coupled with PV formation [43]. Dense granules secrete after the parasite is completely isolated within the PV. The importance of proteolytic processing of microneme proteins during host cell invasion is particularly well documented in *P. falciparum* [37] and *T. gondii* [38]. The processing is thought to be coupled with a timely release, translocation, and shedding of the transmembrane and/or membrane-anchored adhesion proteins that are involved in the host cell invasion. In *P. falciparum*, the microneme proteins such as EBA-175, EBA-140, EBA-181, and AMA-1 are released and/or translocated from the micronemes and shed from the merozoite around the time of RBC invasion [37]. The precise mechanism of parasite ligand shedding remains poorly understood.

Intramembrane proteases including the site-2 protease, a metalloprotease, presenilin and signal peptide peptidase, aspartyl proteases, and rhomboids, a serine protease, have an unusual property of cleaving peptide bonds in the hydrophobic lipid bilayer as enzyme active sites are located within the membrane [44]. The *Drosophila* rhomboid-like intramembrane serine proteases have been identified in the merozoite surface and micronemes of *T. gondii* and *P. falciparum* [38,45–47]. These apicomplexan rhomboids are responsible for the shedding and/or processing of type I integral membrane proteins including *P. falciparum* EBA-175 [47], *P. falciparum* AMA-1 [48], and *T. gondii* AMA-1 [48]. Similar to rhomboids, we believe that a crucial function of PfSPP is to mediate processing of type II integral membrane proteins in the micronemes and on the surface of merozoites in association with their secretion and/or shedding during RBC invasion.

Parasite micronemes and rhoptries are thought to be assembled by the membrane-bound vesicles formed by budding from the Golgi apparatus [49–51]. In *T. gondii*, micronemes discharge their contents by fusing with the apical end of the parasite [52]. Using TgMIC2 as a micronemal protein marker, it was shown that secretion occurs through the extreme apical tip of the parasite at the junction between the parasite and host cell [52]. TgMIC2 was translocated rearwards over the parasite surface and the processing product lacking the cytoplasmic tail was released into the culture supernatant prior to capping. In *T. gondii*, the rhoptries secrete into the host cell cytosol as clusters of vesicles that are deposited into the nascent PV [53]. Although such secretory vesicles have not been identified in *Plasmodia* to our knowledge, it is plausible that the membrane-bound parasite proteins are translocated from the micronemes and rhoptries to the merozoite surface, the PVM, and/or the erythrocyte membrane by means of a vesicular discharge from the secretory organelles. For example, in the *Plasmodium* species, AMA-1 and EBA-175 are believed to play an important role in the RBC invasion, and could translocate from the micronemes to the apical end, merozoite surface,

and/or tight junction by vesicular discharge followed by fusion of the vesicular and plasma membranes.

Major RBC integral membrane proteins including band 3 and glycophorin A are not recruited into the parasitophorous vacuole membrane (PVM) [54], while the PVM lipids primarily originate from the RBC plasma membrane [55]. Following discharge onto the invaginating RBC membrane, the merozoite surface, and the tight junction as a component of the micronemal vesicles, the PfSPP could interact with band 3 in a number of ways. First, PfSPP might be involved in the reorganization of the RBC plasma membrane in recruiting PVM by shedding unnecessary host membrane proteins. Second, PfSPP might play a role in the disassembly of the band 3-MSP1-MSP9 complex [18] by an intramembrane proteolytic cleavage of band 3 or other membrane proteins associated with band 3 in the host plasma membrane. Third, PfSPP might also play a role at the tight junction formation by shedding host and/or parasite-derived type II membrane proteins. In all such cases, the 5ABC domain of band 3 could serve as an effector enhancing the peptidase activity via an allosteric effect by binding to a non-active site in the extracytosolic region of PfSPP.

Our earlier studies suggested that band 3 receptor is involved in the initial attachment of *P. falciparum* merozoites by interacting with MSP1 and MSP9 [17,18]. The prevailing model for RBC invasion by *P. falciparum* [1,2,37] suggests that the interaction between band 3 and PfSPP could occur at a later time point associated with the microneme discharge. To our knowledge, *P. falciparum* PfSPP localized to the micronemes is the first identified apicomplexan protein with an intramembrane aspartyl peptidase activity that binds to a major receptor on erythrocytes. PfSPP could be an essential enzyme since it is the only intramembrane aspartyl protease identified in the *P. falciparum* genome, and is expressed at a relatively high rate throughout the blood-stage life cycle. Identification of the natural substrate(s) of PfSPP could lead to the development of new anti-malarial drugs that would specifically inhibit the unique PfSPP activity. Contingent upon the reactivity of sera from the endemic areas to PfSPP, the inclusion of peptide epitopes of PfSPP in the vicinity of anti-peptide antibody sequence may offer a new option to enhance the efficacy of multi-subunit vaccine against malaria.

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Abbreviations

PBS, phosphate-buffered saline; MBP, maltose binding protein; GST, glutathione S-transferase; KLH, Keyhole Limpet Hemocyanin; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetra acetic acid; Ni-NTA, nickel-nitriloacetic acid agarose; BSA, bovine serum albumin; PfSPP, *Plasmodium falciparum* signal peptide peptidase.

Fig. 1. In silico analysis of *P. falciparum* PfSPP

(A) Phylogenetic tree of *P. falciparum* PfSPP was generated and percent identity was calculated by ClustalW method (MegAlign 5.08, DNASTAR) using the default settings. Protein accession numbers are given in parenthesis. (B) Topology models of PfSPP. The map shows the relative position of predicted signal-anchor sequence (SA; amino acids 19–38), two active site motifs YD (amino acids 221–222) and LGLGD (259–263), and the PALL (335–338) motif.

Transmembrane (TM) regions are shown in black, cytosolic regions in green, and extracytosolic regions in light blue. (a) TMpred model: TM1, amino acids 22–47; TM2, 85–103; TM3, 110–128; TM4, 166–182; TM5, 199–221; TM6, 256–274; TM7, 309–327; TM8, 336–354. (b) TMHMM-2.0 model: TM1, 21–43; TM2, 80–102; TM3, 109–128; TM4, 165–182; TM5, 203–225; TM6, 253–275; TM7, 308–330; TM8, 335–354. (c) Phobius model: TM1, 21–37; TM2, 43–61; TM3, 82–103; TM4, 109–129; TM5, 164–181; TM6, 187–205; TM7, 221–236; TM8, 256–275; TM9, 307–330; TM10, 336–354. (d) SOSUI model: TM1, 31–53; TM2, 80–102; TM3, 108–1030; TM4, 162–184; TM5, 187–209; TM6, 214–236; TM7, 257–279; TM8, 308–329; TM9, 335–354. The region (amino acids 177–406) of PfSPP encoded by the cDNA insert in the yeast two-hybrid screen (Y2H fragment), recombinant PfSPP/ER, and a segment of ER3 used to generate anti-peptide antibodies are shown.

Fig. 2. Native *P. falciparum* PfSPP

(A) Coomassie blue gel shows the affinity-purified recombinant proteins MBP (lane 1) and MBP-PfSPP (lane 2). (B) Characterization of anti-PfSPP Abs. Western blot shows the mono-specific anti-PfSPP Abs reacted specifically to the recombinant PfSPP/ER (lanes 1 and 2) and native *P. falciparum* PfSPP (lanes 4 and 6). Pre-immune controls are shown in lanes 3 and 5. Lane 1, MBP; lanes 2 and 3, MBP-PfSPP/ER; lanes 4 and 5, *P. falciparum* extract; and lane 6, human RBC ghosts.

Fig. 3. Co-localization of PfSPP in mature *P. falciparum*

Indirect immunofluorescence microscopy using specific antibodies showed PfSPP is co-localized with EBA-175 (a microneme protein), but neither with RAP1 (a rhoptry marker) nor MSP1 (merozoite surface protein) in the *P. falciparum* (3D7) schizonts.

Fig. 4. Immunoelectron microscopy

PfSPP was labeled with mono-specific PfSPP Abs using gold particles (15 nm) in *P. falciparum* merozoites. (A) The pre-immune control showed no specific labeling. (B) The anti-PfSPP Abs showed specific labeling of the merozoite with gold particles in the micronemes (arrows), the apical surface area (arrowheads). The parasite nucleus (Nu), rhoptries (Rh), and hemozoin (Hz) are indicated.

Fig. 5. Inhibition of RBC invasion and PfSPP-RBC interactions

(A) The graph shows the rate of inhibition of *P. falciparum* invasion into human RBCs is dependent upon the concentration of anti-PfSPP Abs (●) but not pre-immune IgG (■) present in the culture medium at the time of invasion. The inhibition rate was determined relative to the no-antibody control taken as 100% invasion (0% inhibition). Pre-immune samples showed ~2% inhibition (>98% invasion rate) as compared to the no-antibody control (data not shown). (B) Western blotting using anti-PfSPP Abs shows that a significant amount of full-length PfSPP was found in the preparations of *P. falciparum* culture supernatant separated at 40,000 g for 15 min (lane 1) and 12,000 g for 20 min (lane 2). Lane 3, pellet from 40,000 g centrifugation; lane 4, pellet from 12,000 g centrifugation. An equivalent amount of two supernatant samples

(lanes 1 and 2) and the two pellet samples (lanes 3 and 4) were loaded. **(C)** RBC binding assay in suspension using the culture supernatant prepared by 12,000 *g* centrifugation. Normal (untreated, lane 1), trypsin-treated (lane 4), chymotrypsin-treated (lane 5), and neuraminidase-treated (lane 6) intact human RBC samples were analyzed by Western blotting using anti-PfSPP Abs following the binding assay. Soluble GST-5ABC (lane 3) was added to the binding assay to block the binding of native PfSPP to normal RBCs. GST served as negative control (lane 2). **(D)** Western blotting of samples using anti-PfSPP Abs is shown in each panel. Specific-binding of native PfSPP to recombinant 5ABC domain. Lane 1, *P. falciparum* protein extract prepared using TX-100 (PE); lane 2, PE + GST-5ABC (beads); lane 3, PE + GST (beads); lane 4, GST-5ABC beads only. **(E)** Specific-binding of PfSPP and 5ABC in solution by Western blotting using anti-PfSPP Abs. Lane 1, MBP-PfSPP/ER (beads) + Trx-5ABC; lane 2, MBP (beads) + Trx-5ABC; lane 3, MBP-PfSPP/ER (beads) + Trx.

Fig. 6. A proposed model for the interaction of PfSPP with band 3

The schematic diagram depicts the location of putative binding domains of *P. falciparum* PfSPP and human erythrocyte band 3 as identified in the yeast two-hybrid screen.